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Report

Regulation of host cell cyclin D1 by *Trypanosoma cruzi* in myoblasts

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Infection with the parasite *Trypanosoma cruzi* causes Chagas disease. In this study we demonstrated that there was an increase in cyclin D1 expression in *T. cruzi* (Tulahuen strain)-infected myoblasts. To examine a possible mechanism for the increased cyclin D1 expression we transfected L_6E_9 myoblasts with cyclin D1 luciferase reporter constructs and infected with *T. cruzi*. There was no evidence of an increase in promoter activity. Additionally, quantitative PCR did not demonstrate any change in cyclin D1 message during infection. Moreover, we demonstrated that the cyclin D1 protein was significantly stabilized after infection. Collectively, these data indicate that infection with *T. cruzi* increases cyclin D1 protein abundance post-translationally.

Introduction

Infection with the parasite *Trypanosoma cruzi* causes Chagas disease which is accompanied by acute myocarditis and chronic cardiomyopathy.[1] The parasite readily infects various cell types including cells of the cardiovascular, reticulo-endothelial and autonomic nervous system, as well as skeletal muscle. The consequences of *T. cruzi* infection on host cell cycle regulatory proteins have been extensively investigated both in cultured cells and mouse models.[2-6] Previously we demonstrated that *T. cruzi* infection of CD1 mice resulted in increased cyclin D1 expression, increased ERK activation and increased AP-1 and NFκB DNA binding activity.[4]

As we have previously shown, *cyclin D1* gene expression is regulated in part by transcriptional activation, which can be mediated by the MAPK pathway and NFκB.[7-9] Although both ERK and NFκB are activated by *T. cruzi* infection in endothelial cells[10] the precise mechanism by which cyclin D1 expression is upregulated in this infection is not known and the mode of induction may be cell type specific. In addition, cyclin D1 protein levels can be regulated by post-translational modification, and phosphorylation of cyclin D1 by Glycogen syntase kinase 3β (GSK3β) leads to proteolytic degradation of cyclin D1.[11] In the experiments described herein we demonstrate that infection of L_6E_9 myoblasts with the Tulahuen strain of *T. cruzi* resulted in increased cyclin D1 protein levels. Experiments utilizing both real-time PCR and the cyclin D1 promoter activity assays indicated that the increase in cyclin D1 abundance was not regulated at the transcriptional level in these myoblasts. Interestingly, chemical inhibition of the protein degradation resulted in increased cyclin D1 abundance in infected cells, indicting that *T. cruzi* may affect cell cycle regulation, in part, through stabilization of the cyclin D1 protein in myoblasts.

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Results

***T. cruzi* infection increases cyclin D1 protein levels in infected myoblasts but does not regulate the cyclin D1 promoter.** Immunoblot analyses were performed on infected myoblasts 24 hrs post infection. There was a significant increase in the expression of host cyclin D1 protein levels in lysates of infected myoblasts (Fig. 2A). Myoblasts were transfected for 48 hrs with the luciferase reporter construct of full length (-1745) cyclin D1 gene promoter and infected for 24 hrs as described in the Materials and Methods. Despite the increase in cyclin D1 protein levels, there was no significant change in cyclin D1 promoter activity (Fig. 2B). Similar results were observed using cyclin D1 promoter constructs with deleted AP-1 or ATF2 binding sites (Fig. 2C) and were not affected by either the presence or absence of serum (data not shown).

Quantitative determination of cyclin D1 mRNA. To confirm that the increased levels of cyclin D1 by *T. cruzi* infection was not due to increased cyclin D1 gene transcription, qPCR analysis was performed on infected and uninfected myoblasts. At 24 hrs post infection, qPCR analysis revealed no increase in cyclin D1 mRNA levels compared with uninfected myoblast (Fig. 2D), consistent with the promoter activity data.

Cyclin D1 protein degradation. In order to investigate whether the increase in cyclin D1 protein levels observed following infection

where due to changes in cyclin D1 stability, protein synthesis was inhibited by cyclohexamide and the quantitated protein half-life for cyclin D1 in infected myoblasts was compared with the uninfected control myoblasts. The cyclin D1 $t_{1/2}$ was estimated at 22 min under normal conditions but was substantially increased following *T. cruzi* infection (with $t_{1/2}$ > 1 hr) (Fig. 3).

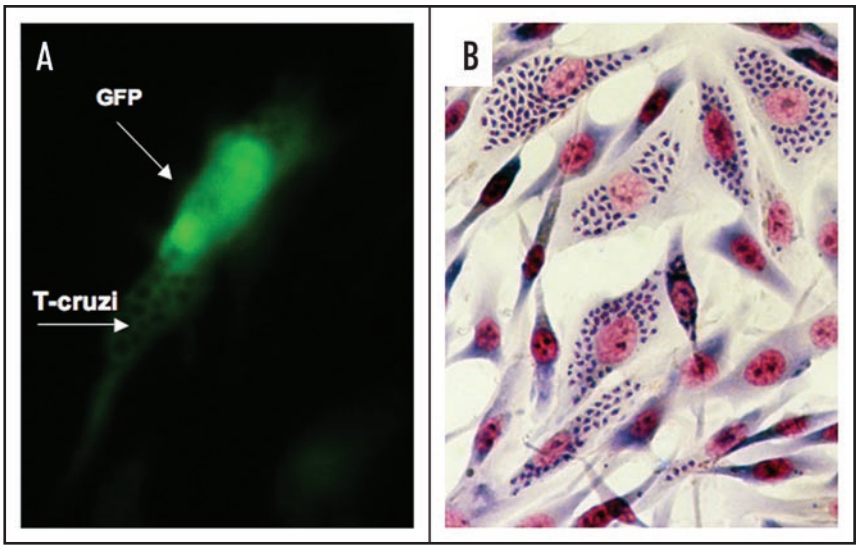

Figure 1. L_6E_9 myoblasts infected with *Trypanosoma cruzi* for 48 hrs. (A) and transfected (B). Note the intracellular amastigotes (arrow).

Discussion

In several in vitro and in vivo studies we have demonstrated that infection with the parasite *T. cruzi*, the causative agent of Chagas disease, resulted in an increased expression of cyclin D1.[2-5] In the present study we reaffirmed in cultured L_6E_9 myoblasts that infection indeed increased the levels of cyclin D1. In order to examine the mechanism by which the parasite causes increased cyclin D1 levels, we used two cyclin D1 reporters which were transfected into myoblasts, either the luciferase reporter construct of the full length (1745) cyclin D1 gene promoter or a cyclin D1 luciferase reporter construct mutated at the AP-1 and ATF binding sites. Surprisingly there were no significant differences in promoter activation either between infected and uninfected control myoblasts, or between the wild type and mutant promoter. Additionally, the presence or absence of serum had no effect on the outcome. Interestingly, qPCR demonstrated that mRNA for cyclin D1 was not increased after infection, supporting the promoter-reporter activity data.

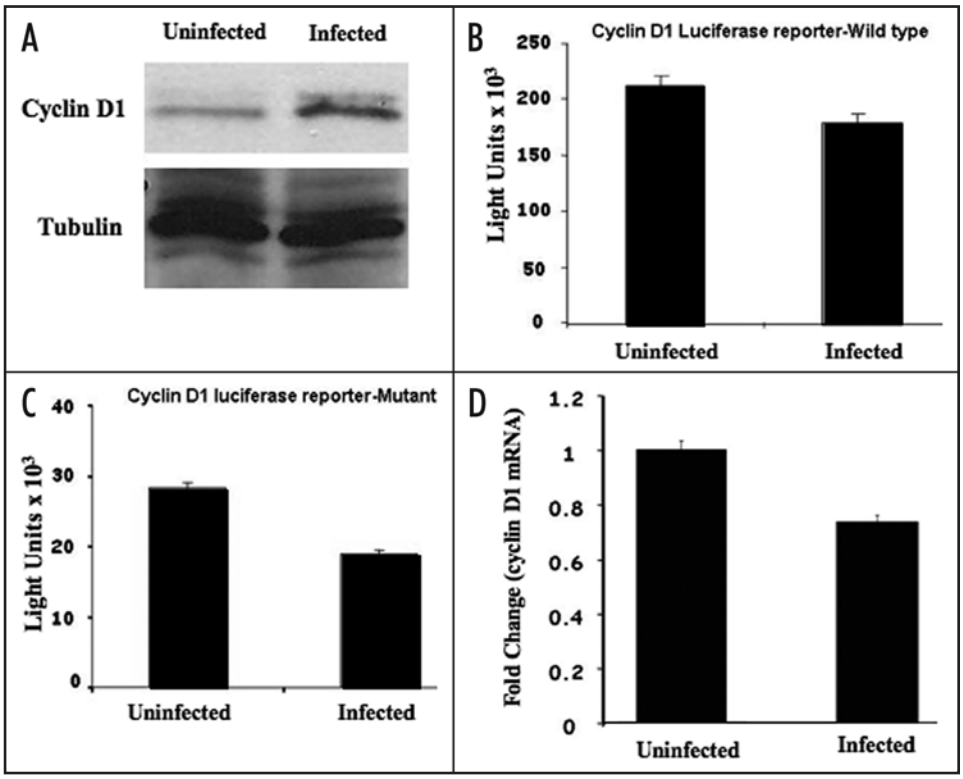

Figure 2. Effect of infection on cyclin D1. (A) Representative cyclin D1 expression in lysates of infected and uninfected L_6E_9 myoblasts by immunoblot analysis. Tubulin was used as a loading control. Effect of *T. cruzi* infection on the wildtype -1745 cyclin D1 LUC (B) and the mutant cyclin D1 promoter (C). Myoblasts were transfected with the cyclin D1 promoter constructs for 24 hrs then infected. (D) Cyclin D1 mRNA levels of infected and uninfected myoblasts 24 hrs post infection.

Using cyclohexamide, an inhibitor of the protein synthesis, we investigated the possibility that infection with *T. cruzi* may alter cyclin D1 protein stability. We observed that the half-life of cyclin D1 was significantly increased in the infected versus uninfected cells. Using MG-132, an inhibitor of the proteasomal pathway, we observed that cyclin D1 was degraded by proteasome-dependent mechanisms in both cases (data not shown), which confirms previous reports that cyclin D1 is degraded by the proteasomal complex.[14,15] Taken together, these observations indicate that the *T. cruzi*-induced increase in cyclin D1 protein levels did not occur at the level of gene transcription but rather was regulated through post-translational mechanisms.

Although cyclin D1 has usually been associated with cell hypertrophy and proliferation, it has recently also been associated with left ventricular hypertrophy and cardiac myocyte proliferation.[16] In previous studies from this laboratory, we have demonstrated that infection of mice with the Brazil strain of *T. cruzi* resulted in a dilated cardiomyopathy as demonstrated by pathologic examination of the heart as well as echocardiography and magnetic resonance imaging.[17,18] As with all dilated cardiomyopathies there was cardiac myocyte hypertrophy. We were the first group to demonstrate that *T. cruzi* infection of mice resulted in increased expression of host cyclin D1 as well as the activation of the ERK signal transduction cascade.[4] For example, in our in vivo experiments, we discovered that the

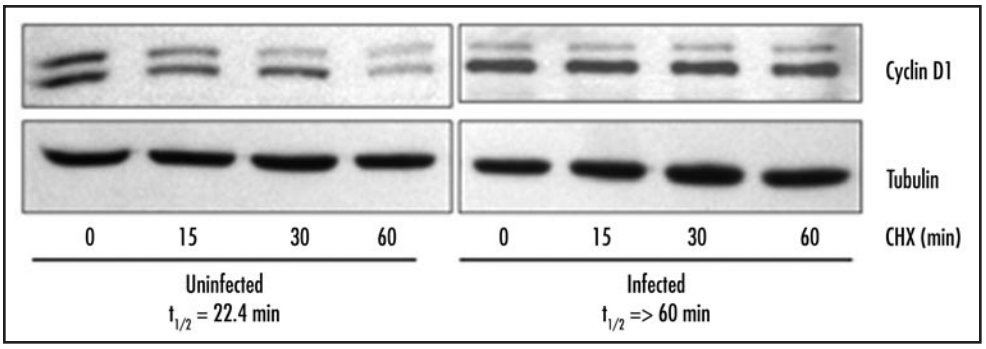

Figure 3. Effect of infection on cyclin D1 protein stability. L_6E_9 myoblasts were infected with trypomastigotes for 48 hrs. Monolayers were washed free of trypomastigotes and treated with cycloheximide (200 μg/ml) for periods of time ranging from 15 min to 1 hr.

hearts quickly displayed an increase in the expression of cyclin D1 as well as other cyclins, such as cyclin E and cyclin A.[2] The induction of cyclin A and cyclin E indicate that a reversion to a fetal or neonatal phenotype may be occurring within the infected heart. The ERK signaling pathway regulates the expression of cyclin D1 and both are negatively regulated by caveolin-1.[19] We have also demonstrated previously that infection caused a reduction in the expression of caveolin-1 both in vitro and in vivo.[2,3,5] Despite these findings, the mechanism(s) by which host cyclin D1 abundance was regulated by *T. cruzi* in the myocardium had not been previously investigated. We anticipated that infection of myoblasts would result in an increase in transcription of cyclin D1, resulting in increased cyclin D1 protein levels. To our surprise we found that while *T. cruzi* infection did indeed significantly increase cyclin D1 protein abundance, there was no significant change in the promoter activity or in mRNA levels. Our data show, for the first time, that infection of L_6E_9 myoblasts with *T. cruzi* results in an increase in cyclin D1 protein stability. While additional investigations will be required to better assess the mechanisms involved in proteasome inhibition during infection, our data provide new insights into possible therapeutic intervention strategies for *T. cruzi*-mediated myocardial disease.

Materials and Methods

Reagents. Anti-cyclin D1 (DSC-6) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-β-tubulin (D66) antibody and cyclohexamide were from Sigma-Aldrich (St. Louis, MO).

Infection of L_6E_9 myoblasts. The Tulahuen strain of *T. cruzi* was maintained in A/J mice (Jackson Laboratories, Bar Harbor, ME) and in L_6E_9 neonatal rat myoblasts as previously described.[12] The myoblasts were grown to near confluency in DMEM (Mediatech, Manassas, VA) supplemented with 15% FBS, Penicillin and Streptomycin. They were then infected with trypomastigotes obtained from infected cultures as previously described[12] and infected at a multiplicity of infection of 5–10:1 for variable incubation times at 37°C and 5% CO_2. Infected cultures were stained with Giemsa (Fig. 1B).

Experimental design. In all infection and transfection experiments, the myoblasts were plated and then transfected on the following day. After 24 hrs of transfection, the myoblasts were infected for an additional 24 hrs and then were harvested. Therefore, at the time of cell harvest, the cells had been transfected for 48 hrs and infected for 24 hrs (Fig. 1A and B).

Immunoblotting. Myoblasts were plated in 100 mm plates and 24 hrs post infection lysates of infected and uninfected cells were prepared in lysis buffer containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1% Tween 20, 0.1 mM PMSF, 2.5 mg/ml leupeptin and 0.1 mM Na3 VO_4 and stored at -80°C until use. Immunoblotting was performed as previously described.[2]

Quantitative determination of cyclins by real time PCR (qPCR). Myoblasts were plated in 100 mm plates and 24 hrs post infection samples were obtained from infected and uninfected L_6E_9 cells and RNA isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA was reverse-transcribed from 5 ng of total RNA in a final volume of 20 μl using Superscript II transcriptase according to the manufacturer's protocol (Invitrogen). The reverse transcription mixture consisted of 0.5 mM dNTPs, 20 mM dithiothritol, 30 mM Tris HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 500 ng oligo dT and 200 U of superscript RT RNase H-reverse transcriptase (Invitrogen). The reactions were incubated for 50 min at 42°C. The qPCR primers for Cyclin D1 were: Forward, 5'-GGGGACAACTCTTAAGTCTCAC- 3' and Reverse, 5'- CCAATAAAAGACCAATCTCTC-3' which amplified a 206-bp cyclin D1 gene fragment. The qPCR was run using 2 mM magnesium chloride and the PCR Syber Green Master Mix (Roche Applied Science, Indianapolis, IN) in a final volume of 20 μl. Reaction mixes were loaded into the Roche Light Cycler Capillaries, capped, centrifuged for 10 s at 2000 rpm and placed in the Light Cycler (Roche Applied Science). The reactions were run at conditions described earlier by Nagajyothi et al.[6] Each run contained a negative control where no DNA was added. To generate PCR standards genomic DNA was isolated from these tissues using the Qiagen DNeasy kit following the manufacturer's protocol (QIAGEN Inc, Valencia, CA). For the quantification of cyclin D1 by qPCR, a standard curve in the range of 5 pg to 50 ng was generated using the primers and conditions listed above. The result was normalized by dividing the number of copies of the target mRNA by the number of copies of microglobulin mRNA for each sample. The primer sequences used for the microglobulin were: Forward, 5'TGGGAAGCCGAACATACTG 3' and Reverse, 5'GCAGGCGTATGTATCAGTCTCA 3' which amplified a 190 bp globulin gene fragment.

Plasmids. Cyclin D1 promoter-luciferase (CD1Luc) reporters, including -1745CD1Luc and cyclin D1 promoter luciferase reporter constructs mutated at the AP-1 and ATF binding sites, were used, as described by Albanese et al.[13]

Transfection and luciferase activity assay. L_6E_9 myoblasts were grown to 60% confluence in 24 well plates and transfected with the human cyclin D1 Luc-reporters using Effectine (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Briefly, reporter DNA (0.1, 0.2 and 0.3 μg) was mixed with the DNA concentration buffer, to a total volume of 150 μl. 8 μl of enhancer was added to this volume and incubated at room temperature for 5 min. Twenty-five μl of Effectine was added to the mixture and the

samples were incubated for 15 min at room temperature to allow transfection-complex formation. Cell culture medium (0.4 ml) was added to the samples and the total mixture was applied to the cells. After 24, 48 and 72 hrs cell extracts were prepared, and the luciferase activities measured. Luciferase assays were performed at room temperature using an Autolumat LB 953 (Berthold Technologies, Oak Ridge, TN). Luciferase content was measured by calculating the light emitted during the initial 10 sec of the reaction and the values are expressed in arbitrary light units.

Degradation of cyclin D1. The rates of degradation of cyclin D1 after infection in myoblasts were determined by treating myoblasts with cycloheximide in order to inhibit protein synthesis. Myoblasts were plated in 60 mm plates and infected with trypomastigotes for 48 hrs. Monolayers were washed free of trypomastigotes and treated with cycloheximide (200 μg/ml) for varying periods of time, ranging from 15 min to 2 hrs. Uninfected cells were used as controls and were treated in an identical fashion. All samples were lysed in RIPA buffer (150 mM NaCl; 50 mM Tris-HCl, pH8.0; 1% NP-40; 0.5% Sodium Deoxycholate; 0.1% SDS) with protease inhibitor cocktail (Roche Applied Sciences) and 20 μg of the total cell lysates were analyzed by immunoblot assays using anti-cyclin D1 antibody. Anti-tubulin antibody was used as a loading control. Images were quantified by densitometry. Statistical analyses were performed using the Mann Whitney U test.

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